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2.	Patent application number (The Patent Office will fill in this part)	9808599.6		
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	Patents ADP number (if you know it)			
	If the applicant is a corporate body, give country/state of incorporation	Norway <i>CG2+Bqg 1001.</i>		
4.	Title of the invention	Improvements in or relating to contrast agents		
5.	Name of your agent (if you have one)	Frank B. Dehn & Co.		
	"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	179 Queen Victoria Street London EC4V 4EL		
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11.

I/We request the grant of a patent on the basis of this application.

Signature

Date 22 April 1998

Frank B. Dehn & Co. - Agents for the applicant

12. Name and daytime telephone number of person to contact in the United Kingdom

J.C. Marsden
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Improvements in or relating to contrast agents

5 This invention relates to ultrasound imaging, more particularly to novel contrast agent preparations and their use in ultrasound imaging, for example in visualising tissue perfusion.

10 It is well known that contrast agents comprising dispersions of microbubbles of gases are particularly efficient backscatterers of ultrasound by virtue of the low density and ease of compressibility of the microbubbles. Such microbubble dispersions, if
15 appropriately stabilised, may permit highly effective ultrasound visualisation of, for example, the vascular system and tissue microvasculature, often at advantageously low doses.

 The use of ultrasonography to measure blood perfusion (i.e. blood flow per unit of tissue mass) is
20 of potential value in, for example, tumour detection, tumour tissue typically having different vascularity from healthy tissue, and studies of the myocardium, e.g. to detect myocardial infarctions. A problem with the application of existing ultrasound contrast agents to
25 cardiac perfusion studies is that the information content of images obtained is degraded by attenuation caused by contrast agent present in the ventricles of the heart.

 In our copending but as yet unpublished
30 International Patent Application No. PCT/GB97/02898, the contents of which are incorporated herein by reference, we have disclosed that ultrasonic visualisation of a subject, in particular of perfusion in the myocardium and other tissues, may be achieved and/or enhanced by
35 means of gas-containing contrast agent preparations which promote controllable and temporary growth of the gas phase *in vivo* following administration. Such

contrast agent preparations may be used to promote controllable and temporary retention of the gas phase, for example in the form of microbubbles, in tissue microvasculature, thereby enhancing the concentration of gas in such tissue and accordingly enhancing its echogenicity, e.g. relative to the blood pool.

Such use of gas as a deposited perfusion tracer differs markedly from existing proposals regarding intravenously administrable microbubble ultrasound contrast agents. Thus it is generally thought necessary to avoid microbubble growth since, if uncontrolled, this may lead to potentially hazardous tissue embolisation. Accordingly it may be necessary to limit the dose administered and/or to use gas mixtures with compositions selected so as to minimise bubble growth *in vivo* by inhibiting inward diffusion of blood gases into the microbubbles (see e.g. WO-A-9503835 and WO-A-9516467).

In accordance with PCT/GB97/02898, on the other hand, a composition comprising a dispersed gas phase is coadministered with a composition comprising at least one substance which has or is capable of generating a gas or vapour pressure *in vivo* sufficient to promote controllable growth of the said dispersed gas phase through inward diffusion thereto of molecules of gas or vapour derived from said substance, which for brevity is hereinafter referred to as a "diffusible component", although it will be appreciated that transport mechanisms other than diffusion may additionally or alternatively be involved in operation of the invention, as discussed in greater detail hereinafter.

This coadministration of a dispersed gas phase-containing composition and a composition comprising a diffusible component having an appropriate degree of volatility may be contrasted with previous proposals regarding administration of volatile substances alone, e.g. in the form of phase shift colloids as described in

WO-A-9416739. Thus the contrast agent preparations of PCT/GB97/02898 permit control of factors such as the probability and/or rate of growth of the dispersed gas by selection of appropriate constituents of the
5 coadministered compositions, whereas administration of the aforementioned phase shift colloids alone may lead to generation of microbubbles which grow uncontrollably and unevenly, possibly to the extent where at least a proportion of the microbubbles may cause potentially
10 dangerous embolisation of, for example, the myocardial vasculature and brain (see e.g. Schwarz, *Advances in Echo-Contrast [1994(3)]*, pp. 48-49).

It has been found that administration of phase shift colloids alone may not lead to reliable or
15 consistent *in vivo* volatilisation of the dispersed phase to generate gas or vapour microbubbles. Grayburn *et al.* in *J. Am. Coll. Cardiol.* **26(5)** [1995], pp. 1340-1347 suggest that preactivation of perfluoropentane emulsions may be required to achieve myocardial opacification in
20 dogs at effective imaging doses low enough to avoid haemodynamic side effects. An activation technique for such colloidal dispersions, involving application of hypobaric forces thereto, is described in WO-A-9640282; typically this involves partially filling a syringe with
25 the emulsion and subsequently forcibly withdrawing and then releasing the plunger of the syringe to generate a transient pressure change which causes formation of gas microbubbles within the emulsion. This is an inherently somewhat cumbersome technique which may fail to give
30 consistent levels of activation.

Again with regard to phase shift colloids, it is stated in US-A-5536489 that emulsions of water-insoluble gas-forming chemicals such as perfluoropentane may be used as contrast agents for site-specific imaging, the
35 emulsions only generating a significant number of image-enhancing gas microbubbles upon application of ultrasonic energy to a specific location in the body

which it is desired to image. Our own research has shown, however, that emulsions of volatile compounds such as 2-methylbutane or perfluoropentane give no detectable echo enhancement either *in vitro* or *in vivo* when ultrasonicated at energy levels which are sufficient to give pronounced contrast effects using two component contrast agents in accordance with PCT/GB97/02898.

The combined preparations of PCT/GB97/02898 are intended for simultaneous, separate or sequential use as a contrast agent in ultrasound imaging, and comprise:

- i) an injectable aqueous composition having gas dispersed therein; and
- ii) a composition comprising a diffusible component capable of diffusion *in vivo* into said dispersed gas so as at least transiently to increase the size thereof.

The preparations may advantageously be employed in visualising tissue perfusion in a subject, the increase in size of the dispersed gas being utilised to effect enrichment or temporary retention of gas in the microvasculature of such tissue, thereby enhancing its echogenicity.

A particular advantage of the preparations is that growth of the dispersed gas may be induced or enhanced by ultrasonication, by application of appropriate amounts of other forms of energy, including sound energy at lower or higher frequencies than those normally used in medical ultrasound imaging, shaking, vibration, an electric field or radiation, or by particle bombardment, for example with neutral particles, ions or electrons. This permits particularly effective control of factors such as the onset and rate of growth of the dispersed gas, and permits such growth to be localised to particular areas of the body of a subject, for example so as to effect temporary retention of gas in the microvasculature of a target organ, e.g. in the

myocardium.

The present invention is based on the finding that the efficacy of contrast agent preparations of the type disclosed in PCT/GB97/02898 may be substantially enhanced if the two compositions are formulated in such a way that the dispersed gas component and diffusible component have affinity for each other, for example as a result of attractive electrostatic or other physical forces or of chemical (including biological) binding. This may be achieved by formulating the dispersed gas component as a stabilised gas dispersion and the diffusible component as a stabilised emulsion such that the stabilising materials for the two components have affinity for each other.

Whilst we do not wish to be bound by theoretical considerations, it is believed that the resulting affinity between the dispersed gas and the diffusible component increases the probability of interaction between them, so that a greater number of dispersed gas moieties are caused to grow for a given dose of the two components compared to the situation where the components lack such mutual affinity. This may particularly be the case where ultrasound or like activation is employed to induce growth of the dispersed gas. Here, in situations where there is no significant affinity between the components, it is thought that ultrasonication may lead to disintegration of a substantial proportion of the dispersed gas phase and only a relatively low level of interaction with diffusible component. The level of interaction may, however, be markedly increased by use of gas and diffusible components with mutual affinity.

Contrast agent preparations according to the invention may therefore be used at significantly lower doses than are suggested in PCT/GB97/02898 whilst giving equivalent contrast effects. This has valuable implications as regards product safety, since it may

permit the use of diffusible component emulsions at such low levels that any risk of embolisation from the volatile content thereof, e.g. as described in *J. Appl. Physiol.* 40(5) [1976], pp. 745-751, is negligible, even
5 after dilution with blood gases.

Furthermore, it has been found that contrast agent preparations according to the invention may readily permit effective imaging of tissue such as the myocardium using conventional B-mode scanning
10 techniques. Thus the ultrasound energy emitted by scanners operating in B-mode is sufficient to induce growth of the dispersed gas phase, which is then retained in the microvasculature and may be capable of generating diagnostically useful information for at
15 least 5-10 minutes without undergoing ultrasound-induced deterioration. Such behaviour is markedly different from that exhibited by existing gas-containing contrast agents, which in general undergo relatively rapid degradation during ultrasonication and may therefore
20 require use of more complex techniques, for example involving intermittent imaging, to effect satisfactory visualisation.

According to one aspect thereof, the present invention provides a combined preparation for
25 simultaneous, separate or sequential use as a contrast agent in ultrasound imaging, said preparation comprising:

- i) a first composition which is an injectable aqueous medium comprising dispersed gas and material
30 serving to stabilise said gas; and
- ii) a second composition which is an injectable oil-in-water emulsion wherein the oil phase comprises a diffusible component capable of diffusion *in vivo* into said dispersed gas so as at least transiently to
35 increase the size thereof, said composition further comprising material serving to stabilise said emulsion, characterised in that said gas-stabilising material

and said emulsion-stabilising material have affinity for each other.

The invention further provides a method of generating enhanced images of a human or non-human animal subject which comprises the steps of:

- i) injecting a first composition as defined above into the vascular system of said subject;
- ii) before, during or after injection of said first composition injecting a second composition as defined above into said subject; and
- iii) generating an ultrasound image of at least a part of said object.

The necessary affinity between the gas-stabilising and emulsion stabilising materials may, for example, be achieved by using materials with opposite charges so that they interact and bind electrostatically to each other. Thus, for example, one of the stabilising materials may be a cationic surfactant and the other an anionic surfactant, e.g. as discussed in greater detail hereinafter. Alternatively, the respective stabilising materials may contain or have attached thereto specific groups, molecules, ligands or vectors capable of interaction through chemical binding interactions such as covalent bonding, hydrogen bonding or ionic bonding. Thus the stabilising materials may, for example, respectively contain or have attached thereto an antigen and an antibody or fragment thereof, a lectin and a carbohydrate-containing group, avidin/streptavidin and biotin or a biotinyl group, a drug and a receptor, a transmitter and a receptor, a hormone and a receptor, a peptide or protein and a complementary peptide or protein, an enzyme or inactive enzyme and a substrate analogue or inhibitor, a nucleic acid (DNA or RNA) sequence and a complementary nucleic acid sequence, or a chelator and a ligand; the foregoing list is not to be considered limiting.

In general any biocompatible gas may be present in

the gas dispersion used as the first composition in accordance with the invention, the term "gas" as used herein including any substances (including mixtures) at least partially, e.g. substantially or completely, in
5 gaseous or vapour form at the normal human body temperature of 37°C. Representative gases thus include air; nitrogen; oxygen; carbon dioxide; hydrogen; inert gases such as helium, argon, xenon or krypton; sulphur fluorides such as sulphur hexafluoride, disulphur
10 decafluoride or trifluoromethylsulphur pentafluoride; selenium hexafluoride; optionally halogenated silanes such as methylsilane or dimethylsilane; low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms), for example alkanes such as methane, ethane, a
15 propane, a butane or a pentane, cycloalkanes such as cyclopropane, cyclobutane or cyclopentane, alkenes such as ethylene, propene, propadiene or a butene, or alkynes such as acetylene or propyne; ethers such as dimethyl ether; ketones; esters; halogenated low molecular weight
20 hydrocarbons (e.g. containing up to 7 carbon atoms); or mixtures of any of the foregoing. Advantageously at least some of the halogen atoms in halogenated gases are fluorine atoms; thus biocompatible halogenated hydrocarbon gases may, for example, be selected from
25 bromochlorodifluoromethane, chlorodifluoromethane, dichlorodifluoromethane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, dichlorotetrafluoroethane, chlorotrifluoroethylene, fluoroethylene, ethylfluoride, 1,1-difluoroethane and
30 perfluorocarbons. Representative perfluorocarbons include perfluoroalkanes such as perfluoromethane, perfluoroethane, perfluoropropanes, perfluorobutanes (e.g. perfluoro-n-butane, optionally in admixture with other isomers such as perfluoro-iso-butane),
35 perfluoropentanes, perfluorohexanes or perfluoroheptanes; perfluoroalkenes such as perfluoropropene, perfluorobutenes (e.g. perfluorobut-2-ene),

perfluorobutadiene, perfluoropentenes (e.g. perfluoropent-1-ene) or perfluoro-4-methylpent-2-ene; perfluoroalkynes such as perfluorobut-2-yne; and perfluorocycloalkanes such as perfluorocyclobutane, perfluoromethylcyclobutane, perfluorodimethylcyclobutanes, perfluorotrimethyl-cyclobutanes, perfluorocyclopentane, perfluoromethyl-cyclopentane, perfluorodimethylcyclopentanes, perfluorocyclohexane, perfluoromethylcyclohexane or perfluorocycloheptane. Other halogenated gases include methyl chloride, fluorinated (e.g. perfluorinated) ketones such as perfluoroacetone and fluorinated (e.g. perfluorinated) ethers such as perfluorodiethyl ether. The use of perfluorinated gases, for example sulphur hexafluoride and perfluorocarbons such as perfluoropropane, perfluorobutanes, perfluoropentanes and perfluorohexanes, may be particularly advantageous in view of the recognised high stability in the bloodstream of microbubbles containing such gases. Other gases with physicochemical characteristics which cause them to form highly stable microbubbles in the bloodstream may likewise be useful.

The gas may, for example, be present in the first composition in the form of microbubbles at least partially encapsulated or otherwise stabilised by the gas-stabilising material. This stabilising material may, for example, comprise a coalescence-resistant surface membrane (for example gelatin, e.g. as described in WO-A-8002365), a filmogenic protein (for example an albumin such as human serum albumin, e.g. as described in US-A-4718433, US-A-4774958, US-A-4844882, EP-A-0359246, WO-A-9112823, WO-A-9205806, WO-A-9217213, WO-A-9406477 or WO-A-9501187), a polymer material (for example a synthetic biodegradable polymer as described in EP-A-0398935, an elastic interfacial synthetic polymer membrane as described in EP-A-0458745, a microparticulate biodegradable polyaldehyde as described

in EP-A-0441468, a microparticulate N-dicarboxylic acid derivative of a polyamino acid - polycyclic imide as described in EP-A-0458079, or a biodegradable polymer as described in WO-A-9317718 or WO-A-9607434), a non-
5 polymeric and non-polymerisable wall-forming material (for example as described in WO-A-9521631), or a surfactant (for example a polyoxyethylene-polyoxypropylene block copolymer surfactant such as a Pluronic, a polymer surfactant as described in WO-A-
10 9506518, or a film-forming surfactant such as a phospholipid, e.g. as described in WO-A-9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780, WO-A-9503835 or WO-A-9729783).

Other useful stabilising materials include gas-
15 containing solid systems, for example microparticles (especially aggregates of microparticles) having gas contained therewithin or otherwise associated therewith (for example being adsorbed on the surface thereof and/or contained within voids, cavities or pores
20 therein, e.g. as described in EP-A-0122624, EP-A-0123235, EP-A-0365467, WO-A-9221382, WO-A-9300930, WO-A-9313802, WO-A-9313808 or WO-A-9313809). It will be appreciated that the echogenicity of such microparticulate contrast agents may derive directly
25 from the contained/associated gas and/or from gas (e.g. microbubbles) liberated from the solid material (e.g. upon dissolution of the microparticulate structure).

The disclosures of all of the above-described documents relating to gas-containing formulations are
30 incorporated herein by reference.

Gas microbubbles and other gas-containing materials such as microparticles preferably have an initial average size not exceeding 10 μm (e.g. of 7 μm or less) in order to permit their free passage through the
35 pulmonary system following administration, e.g. by intravenous injection. However, larger microbubbles may be employed where, for example, these contain a mixture

of one or more relatively blood-soluble or otherwise diffusible gases such as air, oxygen, nitrogen or carbon dioxide with one or more substantially insoluble and non-diffusible gases such as perfluorocarbons. Outward
5 diffusion of the soluble/diffusible gas content following administration will cause such microbubbles rapidly to shrink to a size which will be determined by the amount of insoluble/non-diffusible gas present and which may be selected to permit passage of the resulting
10 microbubbles through the lung capillaries of the pulmonary system.

Since dispersed gas administered in accordance with the invention is caused to grow *in vivo* through interaction with diffusible component, the minimum size
15 of the microbubbles, solid-associated gas etc. as administered may be substantially lower than the size normally thought necessary to provide significant interaction with ultrasound (typically ca. 1-5 μm at conventionally-employed imaging frequencies); the
20 dispersed gas moieties may therefore have sizes as low as, for example, 1 nm or below. The invention may accordingly permit use of gas-containing compositions which have not hitherto been proposed for use as ultrasound contrast agents, e.g. because of the low size
25 of the dispersed gas moieties.

Where phospholipid-containing first compositions are employed in accordance with the invention, e.g. in the form of phospholipid-stabilised gas microbubbles, representative examples of useful phospholipids include
30 lecithins (i.e. phosphatidylcholines), for example natural lecithins such as egg yolk lecithin or soya bean lecithin, semisynthetic (e.g. partially or fully hydrogenated) lecithins and synthetic lecithins such as dimyristoylphosphatidylcholine,
35 dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine; phosphatidic acids; phosphatidylethanolamines; phosphatidylserines;

phosphatidylglycerols; phosphatidylinositols; cardiolipins; sphingomyelins; fluorinated analogues of any of the foregoing; mixtures of any of the foregoing and mixtures with other lipids such as cholesterol. The use of phospholipids predominantly (e.g. at least 75%) comprising molecules individually bearing net overall charge, e.g. negative charge, for example as in naturally occurring (e.g. soya bean or egg yolk derived), semisynthetic (e.g. partially or fully hydrogenated) and synthetic phosphatidylserines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids and/or cardiolipins, for example as described in WO-A-9729783, may be particularly advantageous.

Representative examples of gas-containing microparticulate materials which may be useful in first compositions in accordance with the invention include carbohydrates (for example hexoses such as glucose, fructose or galactose; disaccharides such as sucrose, lactose or maltose; pentoses such as arabinose, xylose or ribose; α -, β - and γ -cyclodextrins; polysaccharides such as starch, hydroxyethyl starch, amylose, amylopectin, glycogen, inulin, pulullan, dextran, carboxymethyl dextran, dextran phosphate, ketodextran, aminoethyldextran, alginates, chitin, chitosan, hyaluronic acid or heparin; and sugar alcohols, including alditols such as mannitol or sorbitol), inorganic salts (e.g. sodium chloride), organic salts (e.g. sodium citrate, sodium acetate or sodium tartrate), X-ray contrast agents (e.g. any of the commercially available carboxylic acid and non-ionic amide contrast agents typically containing at least one 2,4,6-triiodophenyl group having substituents such as carboxyl, carbamoyl, N-alkylcarbamoyl, N-hydroxyalkylcarbamoyl, acylamino, N-alkylacylamino or acylaminomethyl at the 3- and/or 5-positions, as in metrizoic acid, diatrizoic acid, iothalamic acid,

ioxaglic acid, iohexol, iopentol, iopamidol, iodixanol, iopromide, metrizamide, iodipamide, meglumine iodipamide, meglumine acetrizoate and meglumine diatrizoate), and polypeptides and proteins (e.g. 5 gelatin or albumin such as human serum albumin).

Other gas-containing materials which may be useful in first compositions in accordance with the invention include gas-containing material stabilised by metals (e.g. as described in US-A-3674461 or US-A-3528809), 10 gas-containing material stabilised by synthetic polymers (e.g. as described in US-A-3975194 or by Farnand in *Powder Technology* 22 [1979], pp. 11-16), commercially available microspheres of the Expancel® type, e.g. Expancel 551 DE (see e.g. *Eur. Plast. News* 9(5) [1982], 15 p. 39, *Nonwovens Industry* [1981], p. 21 and *Mat. Plast. Elast.* 10 [1980], p. 468), commercially available microspheres of the Ropaque® type (see e.g. *J. Coatings Technol.* 55(707) [1983], p. 79), micro- and nano-sized gas-containing structures such as zeolites, inorganic or 20 organic aerogels, nanosized open void-containing chemical structures such as fullerenes, clathrates or nanotubes (e.g. as described by G.E. Gadd in *Science* 277 (5328) [1997], pp. 933-936), and natural surfactant-stabilised microbubble dispersions (e.g. as described by 25 d'Arrigo in "*Stable Gas-in-Liquid Emulsions, Studies in physical and theoretical chemistry*" 40 - Elsevier, Amsterdam [1986]).

The dispersed oil phase in the second composition of preparations according to the invention may comprise 30 any appropriate diffusible component which is at least partially insoluble in and immiscible with water. The diffusible component in such emulsions is advantageously a liquid at processing and storage temperature, which may for example be as low as -10°C if the aqueous phase 35 contains appropriate antifreeze material, while being a gas or exhibiting a substantial vapour pressure at body temperature. Appropriate compounds may, for example, be

selected from the various lists of emulsifiable low boiling liquids given in the aforementioned WO-A-9416379, the contents of which are incorporated herein by reference. Specific examples of emulsifiable

5 diffusible components include aliphatic ethers such as diethyl ether; polycyclic oils or alcohols such as menthol, camphor or eucalyptol; heterocyclic compounds such as furan or dioxane; aliphatic hydrocarbons, which may be saturated or unsaturated and straight chained or

10 branched, e.g. as in n-butane, n-pentane, 2-methylpropane, 2-methylbutane, 2,2-dimethylpropane, 2,2-dimethylbutane, 2,3-dimethylbutane, 1-butene, 2-butene, 2-methylpropene, 1,2-butadiene, 1,3-butadiene, 2-methyl-1-butene, 2-methyl-2-butene, isoprene, 1-pentene, 1,3-

15 pentadiene, 1,4-pentadiene, butenyne, 1-butyne, 2-butyne or 1,3-butadiyne; cycloaliphatic hydrocarbons such as cyclobutane, cyclobutene, methylcyclopropane or cyclopentane; and halogenated low molecular weight hydrocarbons (e.g. containing up to 7 carbon atoms).

20 Representative halogenated hydrocarbons include dichloromethane, methyl bromide, 1,2-dichloroethylene, 1,1-dichloroethane, 1-bromoethylene, 1-chloroethylene, ethyl bromide, ethyl chloride, 1-chloropropene, 3-chloropropene, 1-chloropropane, 2-chloropropane and t-

25 butyl chloride. Advantageously at least some of the halogen atoms are fluorine atoms, for example as in dichlorofluoromethane, trichlorofluoromethane, 1,2-dichloro-1,2-difluoroethane, 1,2-dichloro-1,1,2,2-tetrafluoroethane, 1,1,2-trichloro-1,2,2-

30 trifluoroethane, 2-bromo-2-chloro-1,1,1-trifluoroethane, 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether, 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, partially fluorinated alkanes (e.g. pentafluoropropanes such as 1H,1H,3H-pentafluoropropane, hexafluorobutanes,

35 nonafluorobutanes such as 2H-nonafluoro-t-butane, and decafluoropentanes such as 2H,3H-decafluoropentane), partially fluorinated alkenes (e.g. heptafluoropentenenes

such as 1H,1H,2H-heptafluoropent-1-ene, and
nonafluorohexenes such as 1H,1H,2H-nonafluorohex-1-ene),
fluorinated ethers (e.g. 2,2,3,3,3-pentafluoropropyl
methyl ether or 2,2,3,3,3-pentafluoropropyl
5 difluoromethyl ether) and, more preferably,
perfluorocarbons. Examples of perfluorocarbons include
perfluoroalkanes such as perfluorobutanes,
perfluoropentanes, perfluorohexanes (e.g. perfluoro-2-
methylpentane), perfluoroheptanes, perfluorooctanes,
10 perfluorononanes and perfluorodecanes;
perfluorocycloalkanes such as perfluorocyclobutane,
perfluorodimethyl-cyclobutanes, perfluorocyclopentane
and perfluoromethylcyclopentane; perfluoroalkenes such
as perfluorobutenes (e.g. perfluorobut-2-ene or
15 perfluorobuta-1,3-diene), perfluoropentenenes (e.g.
perfluoropent-1-ene) and perfluorohexenes (e.g.
perfluoro-2-methylpent-2-ene or perfluoro-4-methylpent-
2-ene); perfluorocycloalkenes such as
perfluorocyclopentene or perfluorocyclopentadiene; and
20 perfluorinated alcohols such as perfluoro-t-butanol.

If desired, the diffusible component may be
formulated as part of a proprietary registered
pharmaceutical emulsion, such as Intralipid®
(Pharmacia).

25 The emulsion-stabilising material may typically
comprise one or more surfactants. It will be
appreciated that the nature of such surfactants may
significantly affect factors such as the rate of growth
of the dispersed gas phase. In general a wide range of
30 surfactants may be useful, for example selected from the
extensive lists given in EP-A-0727225, the contents of
which are incorporated herein by reference.
Representative examples of useful surfactants include
fatty acids (e.g. straight chain saturated or
35 unsaturated fatty acids, for example containing 10-20
carbon atoms) and carbohydrate and triglyceride esters
thereof, phospholipids (e.g. lecithin), fluorine-

containing phospholipids, proteins (e.g. albumins such as human serum albumin), polyethylene glycols, block copolymer surfactants (e.g. polyoxyethylene-polyoxypropylene block copolymers such as Pluronics, 5 extended polymers such as acyloxyacyl polyethylene glycols, for example polyethyleneglycol methyl ether 16-hexadecanoyloxy-hexadecanoate, e.g. wherein the polyethylene glycol moiety has a molecular weight of 2300, 5000 or 10000), fluorine-containing surfactants 10 (e.g. as marketed under the trade names Zonyl and Fluorad, or as described in WO-A-9639197, the contents of which are incorporated herein by reference), and cationic surfactants, for example comprising one or more quaternary ammonium groups and one or more lipid groups 15 such as long chain (e.g. C₁₀₋₃₀) alkyl or alkanoyl groups.

The use of cationic surfactants, for example lipophilised quaternary ammonium salts such as didodecyldimethylammonium bromide or 1,2-distearoyl-3-trimethylammoniumpropane, or positively charged 20 phospholipids (e.g. esters of phosphatidic acids such as dipalmitoylphosphatidic acid or distearoylphosphatidic acid with aminoalcohols such as hydroxyethylene-diamine), may be particularly advantageous when the gas-stabilising material is an anionic surfactant, for 25 example a negatively charged phospholipid such as a naturally occurring (e.g. soya bean- or egg yolk-derived), semisynthetic (e.g. partially or fully hydrogenated) or synthetic phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidic 30 acid or cardiolipin, in view of the consequent electrostatic interaction between the dispersed gas component and the diffusible component. The use of fluorine-containing cationic surfactants, e.g. fluorinated positively charged phospholipids or 35 fluorinated cationic surfactants as marketed under the trade name Zonyl, may also be advantageous.

The second composition may, for example, be

injected intravenously, intramuscularly or subcutaneously; the latter routes may be advantageous where it is desired specifically to limit the effect of the diffusible component to a particular target area of a subject. One example of a composition for subcutaneous injection comprises nanoparticles such as are used for lymph angiography.

The droplet size of emulsions intended for intravenous injection should preferably be less than 10 μm , e.g. less than 7 μm , and greater than 0.1 μm in order to facilitate unimpeded passage through the pulmonary system. It may be advantageous to employ first and second compositions respectively comprising dispersed gas microbubbles and dispersed diffusible droplets which have substantially similar sizes, for example having diameters in the range 1-4, e.g. 2-3 μm .

If desired, the diffusible component may also be formulated as a microemulsion. Such systems are advantageous by virtue of their thermodynamic stability and the fact that the diffusible component is in practice uniformly distributed throughout the aqueous phase; microemulsions therefore have the appearance of solutions but may exhibit the properties of emulsions as regards the partial pressure of the dispersed phase.

As noted above, the invention permits the use of substantially lower doses of diffusible component-containing emulsion than has hitherto been thought necessary. Phase shift colloid contrast agents such as are disclosed in WO-A-9416739 are typically administered in amounts corresponding to ca. 0.1 ml dispersed phase/kg body weight. It is stated in PCT/GB97/02898 that where the diffusible component is a perfluorocarbon formulated as an oil-in-water emulsion this may typically be administered at a dose corresponding to 0.2-1.0 μl perfluorocarbon/kg body weight. The present invention, however, permits images comparable to those observed in PCT/GB97/02898 to be obtained using at least

20-fold and possibly up to 200-fold lower doses of diffusible component, for example in the range 1-100 nl diffusible component/kg body weight, e.g. 20 nl diffusible component/kg body weight.

5 Whilst the diffusible component content of emulsions has the capability for at least a 100-fold increase in volume when evaporated, it will be appreciated that at such doses the total administered amount of diffusible component will in general be
10 insufficient to give rise to risks of embolism. Moreover, it is likely that such doses are below any threshold at which gas bubbles might spontaneously be generated in low-pressure venous compartments of the circulation (e.g. the vena cava, right heart chambers
15 and pulmonary artery) as a result of the volatile diffusible component and blood gases supersaturating the blood.

 In order to ensure maximum volatilisation of the diffusible component following administration and to
20 enhance growth of the dispersed gas, both of which are endothermic processes, it may be advantageous to manipulate the temperature of the first and/or the second composition prior to administration and/or to incorporate exothermically reactive constituents
25 therein; the use of such constituents which react exothermically under the influence of ultrasound radiation may be particularly advantageous.

 Growth of the dispersed gas phase *in vivo* may, for example, be accompanied by expansion of any
30 encapsulating stabilising material (where this has sufficient flexibility) and/or by abstraction of excess surfactant or other stabilising material, e.g. from the second composition, to the growing gas-liquid interfaces. It is also possible, however, that
35 stretching of the encapsulating material and/or interaction of the material with ultrasound may substantially increase its porosity. Whereas such

disruption of encapsulating material has hitherto in many cases been found to lead to rapid loss of echogenicity through outward diffusion and dissolution of the gas thereby exposed, we have found that when
5 using contrast agent preparations in accordance with the present invention, the exposed gas exhibits substantially stability. Whilst not wishing to be bound by theoretical calculations, we believe that the exposed gas, e.g. in the form of liberated microbubbles, may be
10 stabilised, e.g. against collapse of the microbubbles, by the supersaturated environment generated by the diffusible component, which provides an inward pressure gradient to counteract the outward diffusive tendency of the microbubble gas. The exposed gas surface, by virtue
15 of the substantial absence of encapsulating material, may cause the contrast agent preparation to exhibit exceptionally favourable acoustic properties as evidenced by high backscatter and low energy absorption (e.g. as expressed by high backscatter: attenuation
20 ratios); this echogenic effect may continue for a significant period, even during continuing ultrasound irradiation.

The stabilising effect of coadministered diffusible component may therefore be used to great advantage to
25 enhance both the duration and magnitude of the echogenicity of existing gas-containing contrast agent formulations in cases where these parameters may be insufficient when the contrast agent composition is administered alone. Thus, for example, the duration of
30 effect of albumin-based contrast agents is often severely limited by collapse of the encapsulating albumin material, either as a result of systolic pressure changes in the heart or venous system or as a consequence of ultrasound irradiation, but may be
35 substantially enhanced by coadministration with a diffusible component in accordance with the present invention.

In a representative embodiment of the method of the invention a composition comprising a dispersed gas component and a composition comprising an emulsified diffusible component are selected such that, following intravenous injection of the two compositions, at least a proportion of the dispersed gas passes through the lungs and then undergoes rapid growth following passage from the lungs through inward diffusion of the diffusible component, so as temporarily to be retained in the myocardium and thereby permit ultrasonic visualisation of myocardial perfusion. As the concentration of volatile diffusible component in the bloodstream falls away, e.g. as the component is cleared from the blood, for example by removal through the lungs and exhalation by the subject, by metabolism or by redistribution to other tissues, the diffusible component will typically diffuse out of the dispersed gas, which will therefore shrink towards its initial smaller size, and ultimately once more becoming free flowing in the bloodstream, typically being removed therefrom by the reticuloendothelial system. This pattern of a substantial transient increase in echogenicity followed by disappearance of contrast effect is markedly different from any echogenic properties exhibited by either of the two compositions when administered alone. It will be appreciated from the foregoing that control of the duration of retention of the dispersed gas may therefore be achieved by appropriate adjustment of the dose and/or formulation of the diffusible component, particularly of the nature and degree of affinity between the gas component and diffusible component.

Other capillary systems, such as but not limited to those of the kidney, liver, spleen, thyroid, skeletal muscle, breast and prostate, may similarly be imaged.

In general, the rate and/or extent of growth of the dispersed gas may be controlled by appropriate selection

of the gas and the gas-stabilising material and, more particularly, the nature of the emulsified diffusible component and the manner in which it is formulated, including the nature of the emulsion-stabilising material and the size of the emulsion droplets. In this last context, for a given amount of emulsified diffusible component, a reduction in droplet size may enhance the rate of transfer of diffusible component relative to that from larger droplets since more rapid release may occur from smaller droplets having higher surface area:volume ratios. Other parameters permitting control include the relative amounts in which the two compositions are administered and, where these are administered separately, the order of administration, the time interval between the two administrations, and possible spatial separation of the two administrations. In this last respect it will be appreciated that the inherent diffusivity of the diffusible component may permit its application to different parts of the body in a wide variety of ways, for example subcutaneously, intravenously or intramuscularly.

Particularly important parameters with regard to the diffusible component are its solubility in water/blood and its diffusibility (e.g. as expressed by its diffusion constants), which will determine its rate of transport through the carrier liquid or blood, and its permeability through any membrane of stabilising material encapsulating the dispersed gas. The pressure generated by the diffusible component *in vivo* will also affects its rate of diffusion into the dispersed gas, as will its concentration. Thus, in accordance with Fick's law, the concentration gradient of diffusible component relative to the distance between, for example, individual gas microbubbles and emulsion droplets, together with the diffusion coefficient of the diffusible substance in the surrounding liquid medium, will determine the rate of transfer by simple diffusion;

the concentration gradient is determined by the solubility of the diffusible component in the surrounding medium and the distance between individual gas microbubbles and emulsion droplets.

5 The effective rate of transport of the diffusible component may, if desired, be controlled by adjusting the viscosity of the dispersed gas phase composition and/or the diffusible component composition, for example by incorporating one or more biocompatible viscosity
10 enhancers such as X-ray contrast agents, polyethylene glycols, carbohydrates, proteins, polymers or alcohols into the formulation. It may, for example, be advantageous to coinject the two compositions as a relatively high volume bolus (e.g. having a volume of at
15 least 20 ml in the case of a 70 kg human subject), since this will delay complete mixing of the constituents with blood (and thus the onset of growth of the dispersed gas) until after entry into the right ventricle of the heart and the lung capillaries. The delay in growth of
20 the dispersed gas may be maximised by employing carrier liquid which is undersaturated with respect to gases and any other diffusible components as hereinbefore defined, e.g. as a result of being cooled.

 As noted above, transport mechanisms other than
25 diffusion may be involved in operation of the invention. Thus, for example, transport may also occur through hydrodynamic flow within the surrounding liquid medium; this may be important in vessels and capillaries where high shear rate flow may occur. Transport of diffusible
30 component to the dispersed gas may also occur as a result of collision or near-collision processes, e.g. between gas microbubbles and emulsion droplets, for example leading to adsorption of diffusible component at the microbubble surface and/or penetration of diffusible
35 component into the microbubble, i.e. a form of coalescence. In such cases the diffusion coefficient and solubility of the diffusible component have a

minimal effect on the rate of transfer, the particle size of the diffusible component (e.g. the droplet size where this is formulated as an emulsion) and the collision frequency between microbubbles and droplets being the principal factors controlling the rate and extent of microbubble growth. Thus, for example, for a given amount of emulsified diffusible component, a reduction in droplet size will lead to an increased overall number of droplets and so may enhance the rate of transfer by reducing the mean interparticle distance between the gas microbubbles and emulsion droplets and thus increasing the probability of collision and/or coalescence. As noted above, the rate of transfers proceeding through collision processes may be markedly enhanced if additional oscillatory movement is imparted to the gas microbubbles and emulsion droplets of the diffusible component through application of ultrasonic energy. The kinetics of collision processes induced by such ultrasonic energy may differ from the kinetics for transport of diffusible component in carrier liquid and/or blood, for example in that specific energy levels may be necessary to initiate coalescence of colliding gas microbubbles and emulsion droplets. Accordingly it may be advantageous to select the size and therefore the mass of the emulsion droplets so that they generate sufficient collisional force with the oscillating microbubbles to induce coalescence.

As also noted above, the permeability of any stabilising material encapsulating the dispersed gas phase is a parameter which may affect the rate of growth of the gas phase, and it may therefore be desirable to select a diffusible component which readily permeates any such encapsulating material (which may, for example, be a polymer or surfactant membrane, e.g. a monolayer or one or more bilayers of a membrane-forming surfactant such as a phospholipid). We have found, however, that substantially impermeable encapsulating material may

also be used, since it appears that sonication- or other energy input-induced growth of the dispersed gas may occur despite the presence of such impermeable material.

5 Whilst we do not wish to be bound by theoretical considerations it may be that ultrasonication at least transiently modifies the permeability of the encapsulating material, the diffusibility of the diffusible component in the surrounding liquid phase and/or the frequency of collisions between emulsion
10 droplets and the encapsulated microbubbles. Since the effect may be observed using extremely short ultrasound pulses (e.g. with durations of ca. 0.3 μ s in B-mode imaging or ca. 2 μ s in Doppler or second harmonic imaging) it seems unlikely to be an example of rectified
15 diffusion, in which ongoing ultrasound irradiation produces a steady increase in the equilibrium radii of gas bubbles (see Leighton, E.G. - *"The Acoustic Bubble"*, Academic Press [1994], p. 379), and it may be that the ultrasound pulses disrupt the encapsulating membrane and
20 so enhance growth of the dispersed gas through inward diffusion of diffusible component into the thus-exposed gas phase.

 If desired, either the dispersed gas or the diffusible component may comprise an azeotropic mixture
25 or may be selected so that an azeotropic mixture is formed *in vivo* as the diffusible component mixes with the dispersed gas. Such azeotrope formation may, for example, be used effectively to enhance the volatility of relatively high molecular weight compounds, e.g.
30 halogenated hydrocarbons such as fluorocarbons (including perfluorocarbons) which under standard conditions are liquid at the normal human body temperature of 37°C, such that they may be administered in gaseous form at this temperature. This has
35 substantial benefits as regards the effective echogenic lifetime *in vivo* of contrast agents containing such azeotropic mixtures since it is known that parameters

such as the water solubility, fat solubility, diffusibility and pressure resistivity of compounds such as fluorocarbons decrease with increasing molecular weight.

5 In general, the recognised natural resistance of azeotropic mixtures to separation of their constituents will enhance the stability of contrast agent components containing the same, both during preparation, storage and handling and following administration.

10 Azeotropic mixtures useful in accordance with the invention may, for example, be selected by reference to literature relating to azeotropes, by experimental investigation and/or by theoretical predictions, e.g. as described by Tanaka in *Fluid Phase Equilibria* 24 (1985),
15 pp. 187-203, by Kittel, C. and Kroemer, H. in Chapter 10 of *Thermal Physics* (W.H. Freeman & Co., New York, USA, 1980) or by Hemmer, P.C. in Chapters 16-22 of *Statistisk Mekanikk* (Tapir, Trondheim, Norway, 1970), the contents of which are incorporated herein by reference.

20 One literature example of an azeotrope which effectively reduces the boiling point of the higher molecular weight component to below normal body temperature is the 57:43 w/w mixture of 1,1,2-trichloro-1,2,2-trifluoromethane (b.p. 47.6°C) and 1,2-difluoro-
25 methane (b.p. 29.6°C) described in US-A-4055049 as having an azeotropic boiling point of 24.9°C. Other examples of halocarbon-containing azeotropic mixtures are disclosed in EP-A-0783017, US-A-5599783, US-A-5605647, US-A-5605882, US-A-5607616, US-A-5607912, US-A-
30 5611210, US-A-5614565 and US-A-5616821, the contents of which are incorporated herein by reference.

 Simons et al. in *J. Chem. Phys.* 18(3) (1950), pp. 335-346 report that mixtures of perfluoro-n-pentane (b.p. 29°C) and n-pentane (b.p. 36°C) exhibit a large
35 positive deviation from Raoult's law; the effect is most pronounced for approximately equimolar mixtures. In practice the boiling point of the azeotropic mixture has

been found to be about 22°C or less. Mixtures of perfluorocarbons and unsubstituted hydrocarbons may in general exhibit useful azeotropic properties; strong azeotropic effects have been observed for mixtures of such components having substantially similar boiling points. Examples of other perfluorocarbon:hydrocarbon azeotropes include mixtures of perfluoro-*n*-hexane (b.p. 59°C) and *n*-pentane, where the azeotrope has a boiling point between room temperature and 35°C, and of perfluoro-4-methylpent-2-ene (b.p. 49°C) and *n*-pentane, where the azeotrope has a boiling point of approximately 25°C.

Other potentially useful azeotropic mixtures include mixtures of halothane and diethyl ether and mixtures of two or more fluorinated gases, for example perfluoropropane and fluoroethane, perfluoropropane and 1,1,1-trifluoroethane, or perfluoroethane and difluoromethane.

It is known that fluorinated gases such as perfluoroethane may form azeotropes with carbon dioxide (see e.g. WO-A-9502652). Accordingly, administration of contrast agents containing such gases may lead to *in vivo* formation of ternary or higher azeotropes with blood gases such as carbon dioxide, thereby further enhancing the stability of the dispersed gas.

Where the two compositions of combined contrast agent preparations according to the invention are to be administered simultaneously they may, for example, be injected from separate syringes via suitable coupling means or may be premixed, preferably under controlled conditions such that premature growth of the dispersed gas is avoided.

Compositions intended for mixing prior to simultaneous administration may advantageously be stored in appropriate dual or multi-chamber devices. Thus, for example, the dispersed gas-containing first composition or a dried precursor therefor [e.g. comprising a

lyophilised residue of a suspension of gas microbubbles in an amphiphilic material-containing aqueous medium, particularly wherein the amphiphilic material consists essentially of phospholipid predominantly (e.g. at least 5 75%, preferably substantially completely) comprising molecules which individually have an overall net (e.g. negative) charge] may be contained in a first chamber such as a vial, to which a syringe containing the diffusible component-containing second composition is 10 sealing connected; the syringe outlet is closed, e.g. with a membrane or plug, to avoid premature mixing. Operation of the syringe plunger ruptures the membrane and causes the second composition to mix with the first composition or to mix with and reconstitute a precursor therefor; following any necessary or desired shaking 15 and/or dilution, the mixture may be withdrawn (e.g. by syringe) and administered.

Alternatively the two compositions may be stored within a single sealed vial or syringe, being separated 20 by, for example, a membrane or plug; an overpressure of gas or vapour may be applied to either or both compositions. Rupture of the membrane or plug, e.g. by insertion of a hypodermic needle into the vial, leads to mixing of the compositions; this may if desired be 25 enhanced by hand-shaking, whereafter the mixture may be withdrawn and administered. Other embodiments, for example in which a vial containing a dried precursor for the first composition is fitted with a first syringe containing a redispersion fluid for said precursor and a 30 second syringe containing the second composition, or in which a vial containing membrane-separated second composition and dried precursor for the first composition is fitted with a syringe containing redispersion fluid for the latter, may similarly be 35 used.

In embodiments of the invention in which the two compositions are mixed prior to administration, either

at the manufacturing stage or subsequently, the mixture will typically be stored at elevated pressure or reduced temperature such that the pressure of the diffusible component is insufficient to provide growth of the dispersed gas. Activation of growth of the dispersed gas may be induced simply by release of excess pressure or by the heating to body temperature which will follow administration of the mixture, or it may if desired be brought about by preheating the mixture immediately before administration.

In embodiments of the invention in which the two compositions are administered separately, the timing between the two administrations may be used to influence the area of the body in which growth of the dispersed gas phase predominantly occurs. Thus, for example, the second composition may be injected first and the diffusible component allowed to concentrate in the liver, thereby enhancing imaging of that organ upon subsequent injection of the dispersed gas-containing first composition. Where the stability of the gas dispersion permits, this may likewise be injected first and allowed to concentrate in the liver, with the diffusible component-containing second composition then being administered to enhance the echogenicity thereof.

Imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging, Doppler amplitude imaging and combinations of these last two techniques with any of the other modalities described above. For a given dose of the gas

dispersion and diffusible component compositions, the use of colour Doppler imaging ultrasound to induce growth of the dispersed gas has been found to give stronger contrast effects during subsequent B-mode
5 imaging, possibly as a result of the higher ultrasound intensities employed. To reduce the effects of movement, successive images of tissues such as the heart or kidney may be collected with the aid of suitable synchronisation techniques (e.g. gating to the ECG or
10 respiratory movement of the subject). Measurement of changes in resonance frequency or frequency absorption which accompany growth of the dispersed gas may also usefully be made to detect the contrast agent.

It will be appreciated that the dispersed gas
15 content of combined contrast agent preparations according to the invention will tend to be temporarily retained in tissue in concentrations proportional to the regional rate of tissue perfusion. Accordingly, when using ultrasound imaging modalities such as conventional
20 or harmonic B-mode imaging where the display is derived directly from return signal intensities, images of such tissue may be interpreted as perfusion maps in which the displayed signal intensity is a function of local perfusion. This is in contrast to images obtained using
25 free-flowing contrast agents, where the regional concentration of contrast agent and corresponding return signal intensity depend on the actual blood content rather than the rate of perfusion of local tissue.

In cardiac studies, where perfusion maps are
30 derived from return signal intensities in accordance with this embodiment of the invention, it may be advantageous to subject a patient to physical or pharmacological stress in order to enhance the distinction, and thus the difference in image
35 intensities, between normally perfused myocardium and any myocardial regions supplied by stenotic arteries. As is known from radionuclide cardiac imaging, such

stress induces vasodilatation and increased blood flow in healthy myocardial tissue, whereas blood flow in underperfused tissue supplied by a stenotic artery is substantially unchanged since the capacity for arteriolar vasodilatation is already exhausted by inherent autoregulation seeking to increase the restricted blood flow.

The application of stress as physical exercise or pharmacologically by administration of adrenergic agonists may cause discomfort such as chest pains in patient groups potentially suffering from heart disease, and it is therefore preferable to enhance the perfusion of healthy tissue by administration of a vasodilating drug, for example selected from adenosine, dipyridamole, nitroglycerine, isosorbide mononitrate, prazosin, doxazosin, dihydralazine, hydralazine, sodium nitroprusside, pentoxifylline, amelodipine, felodipine, isradipine, nifedipine, nimodipine, verapamil, diltiazem and nitrous oxide. In the case of adenosine this may lead to in excess of fourfold increases in coronary blood flow in healthy myocardial tissue, greatly increasing the uptake and temporary retention of contrast agents in accordance with the invention and thus significantly increasing the difference in return signal intensities between normal and hypoperfused myocardial tissue. Because an essentially physical entrapment process is involved, retention of contrast agents according to the invention is highly efficient; this may be compared to the uptake of radionuclide tracers such as thallium 201 and technetium sestamibi, which is limited by low contact time between tracer and tissue and so may require maintenance of vasodilatation for the whole period of blood pool distribution for the tracer (e.g. 4-6 minutes for thallium scintigraphy) to ensure optimum effect. The contrast agents of the invention, on the other hand, do not suffer such diffusion or transport limitations, and since their

retention in myocardial tissue may also rapidly be terminated, for example by cessation of growth-generating ultrasound irradiation, the period of vasodilatation needed to achieve cardiac perfusion imaging in accordance with this embodiment of the invention may be very short, for example less than one minute. This will reduce the duration of any possible discomfort caused to patients by administration of vasodilator drugs.

In view of the fact that the required vasodilatation need only be short lasting, adenosine is a particularly useful vasodilating drug, being both an endogenous substance and having a very short-lasting action as evidenced by a blood pool half-life of only a few seconds. Vasodilatation will accordingly be most intense in the heart, since the drug will tend to reach more distal tissues in less than pharmacologically active concentrations. It will be appreciated that because of this short half-life, repeated injection or infusion of adenosine may be necessary during cardiac imaging in accordance with this embodiment of the invention; by way of example, an initial administration of 150 $\mu\text{g/kg}$ of adenosine may be made substantially simultaneously with administration of the contrast agent composition, followed 10 seconds later by slow injection of a further 150 $\mu\text{g/kg}$ of adenosine, e.g. over a period of 20 seconds. An infusion of adenosine at a constant rate during the time interval covering injection and deposition of contrast agent in the myocardium may also be used.

Contrast agent preparations in accordance with the invention may advantageously be employed as delivery agents for bioactive moieties such as therapeutic drugs (i.e. agents having a beneficial effect on a specific disease in a living human or non-human animal), particularly to targeted sites. Thus, for example, therapeutic compounds may be present in the first

composition, e.g. in the dispersed gas, linked to part
of the stabilising material (e.g. through covalent or
ionic bonds, if desired through a spacer arm), or
physically mixed into such stabilising material; this
5 last option is particularly applicable where the
therapeutic compound and stabilising material have
similar polarities or solubilities.

The controllable growth properties of the dispersed
gas may be utilised to bring about its temporary
10 retention in the microvasculature of a target region of
interest; use of ultrasonic irradiation to induce growth
and thus retention of the gas and associated therapeutic
compound in a target structure is particularly
advantageous. Localised injection of the gas-containing
15 first composition or, more preferably, the diffusible
component-containing second composition, e.g. as
hereinbefore described, may also be used to concentrate
growth of the dispersed gas in a target area.

The therapeutic compound, which may if desired be
20 coupled to a site-specific vector having affinity for
specific cells, structures or pathological sites, may be
released as a result of, for example, stretching or
fracture of the gas-stabilising material caused by
growth of the dispersed gas, solubilisation of the
25 stabilising material, or disintegration of gas-
microbubbles or gas-containing microparticles (e.g.
induced by ultra-sonication or by a reversal of the
concentration gradient of the diffusible component in
the target area). Where a therapeutic agent is
30 chemically linked to the gas-stabilising material, the
linkage or any spacer arm associated therewith may
advantageously contain one or more labile groups which
are cleavable to release the agent. Representative
cleavable groups include amide, imide, imine, ester,
35 anhydride, acetal, carbamate, carbonate, carbonate ester
and disulphide groups which are biodegradable *in vivo*,
e.g. as a result of hydrolytic and/or enzymatic action.

Representative and non-limiting examples of drugs useful in accordance with this embodiment of the invention include antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan, chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (antinomycin D), daunorubicin, doxorubicin hydrochloride, taxol, plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase), etoposide, interferon a-2a and 2b, blood products such as hematoporphyrins or derivatives of the foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine, miconazole or amphotericin B; hormones or hormone analogues such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate, dexamethasone, flunisolide, hydrocortisone, methylprednisolone, paramethasone acetate, prednisolone, prednisone, triamcinolone or fludrocortisone acetate; vitamins such as cyanocobalamin or retinoids; enzymes such as alkaline phosphatase or manganese superoxide dismutase; antiallergic agents such as amlexanox; anticoagulation agents such as warfarin, phenprocoumon or heparin; antithrombotic agents; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as p-aminosalicylic acid, isoniazid, capreomycin sulfate, cycloserine, ethambutol, ethionamide, pyrazinamide, rifampin or streptomycin sulphate; antivirals such as acyclovir, amantadine, azidothymidine, ribavirin or vidarabine; blood vessel dilating agents such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide

dinitrate, nitroglycerin or pentaerythritol
tetranitrate; antibiotics such as dapsone,
chloramphenicol, neomycin, cefaclor, cefadroxil,
cephalexin, cephradine, erythromycin, clindamycin,
5 lincomycin, amoxicillin, ampicillin, bacampicillin,
carbenicillin, dicloxacillin, cyclacillin,
picloxacillin, hetacillin, methicillin, nafcillin,
penicillin or tetracycline; antiinflammatories such as
diflunisal, ibuprofen, indomethacin, meclufenamate,
10 mefenamic acid, naproxen, phenylbutazone, piroxicam,
tolmetin, aspirin or salicylates; antiprotozoans such as
chloroquine, metronidazole, quinine or meglumine
antimonate; antirheumatics such as penicillamine;
narcotics such as paregoric; opiates such as codeine,
15 morphine or opium; cardiac glycosides such as
deslaneside, digitoxin, digoxin, digitalin or digitalis;
neuromuscular blockers such as atracurium mesylate,
gallamine triethiodide, hexafluorenum bromide,
metocurine iodide, pancuronium bromide, succinylcholine
20 chloride, tubocurarine chloride or vecuronium bromide;
sedatives such as amobarbital, amobarbital sodium,
apropbarbital, butabarbital sodium, chloral hydrate,
ethchlorvynol, ethinamate, flurazepam hydrochloride,
glutethimide, methotrimeprazine hydrochloride,
25 methyprylon, midazolam hydrochloride, paraldehyde,
pentobarbital, secobarbital sodium, talbutal, temazepam
or triazolam; local anaesthetics such as bupivacaine,
chloroprocaine, etidocaine, lidocaine, mepivacaine,
procaine or tetracaine; general anaesthetics such as
30 droperidol, etomidate, fentanyl citrate with droperidol,
ketamine hydrochloride, methohexital sodium or
thiopental and pharmaceutically acceptable salts (e.g.
acid addition salts such as the hydrochloride or
hydrobromide or base salts such as sodium, calcium or
35 magnesium salts) or derivatives (e.g. acetates) thereof;
and radiochemicals, e.g. comprising beta-emitters. Of
particular importance are antithrombotic agents such as

heparin and agents with heparin-like activity such as antithrombin III, dalteparin and enoxaparin; blood platelet aggregation inhibitors such as ticlopidine, aspirin, dipyridamole, iloprost and abciximab; and
5 thrombolytic enzymes such as streptokinase and plasminogen activator. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain proteins
10 may be used in the treatment of many different types of diseases. For example, tumour necrosis factor or interleukin-2 may be provided to treat advanced cancers; thymidine kinase may be provided to treat ovarian cancer or brain tumors; interleukin-2 may be provided to treat
15 neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 may be provided to treat cancer.

Contrast agent preparations in accordance with the invention may be used as vehicles for contrast-enhancing moieties for imaging modalities other than ultrasound,
20 for example X-ray, light imaging, magnetic resonance and, more preferably, scintigraphic imaging agents. Controlled growth of the dispersed gas phase may be used to position such agents in areas of interest within the bodies of subjects, for example using ultrasound
25 irradiation of a target organ or tissue to induce the desired controlled growth and temporary retention of the agent, which may then be imaged using the appropriate non-ultrasound imaging modality.

Contrast agent preparations in accordance with the invention may also be used as vehicles for
30 therapeutically active substances which do not necessarily require release from the preparation in order to exhibit their therapeutic effect. Such preparations may, for example, incorporate radioactive
35 atoms or ions such as beta-emitters which exhibit a localised radiation-emitting effect following growth of the dispersed gas phase and temporary retention of the

agent at a target site. It will be appreciated that such agents should preferably be designed so that subsequent shrinkage and cessation of retention of the dispersed gas does not occur until the desired
5 therapeutic radiation dosage has been administered.

Contrast agent preparations in accordance with the invention may additionally exhibit therapeutic properties in their own right. Thus, for example, preparations may be used therapeutically by
10 intravenously injecting a high dose of the agent and then exposing an artery leading to a tumour to local ultrasound irradiation. The growing gas phase may then block blood circulation to the tumour. Thus it is possible by applying localised ultrasonic energy to
15 obtain a controlled and localised embolism; this may be of importance as such or in combination with other therapeutic measures. Concentrations of dispersed gas in capillaries may also enhance absorption of ultrasonic energy in hyperthermic therapy; this may be used in, for
20 example, treatment of liver tumours. Other tissues which may be treated in this way include breast, thyroid and prostate. Irradiation with a relatively high energy (e.g. 5 W) focused ultrasound beam, e.g. at 1.5 MHz, may be appropriate in such applications.

25 The following non-limitative Examples serve to illustrate the invention.

Preparation 1

Perfluorobutane gas dispersion with negatively charged gas-stabilising material

5 Hydrogenated phosphatidylserine (5 mg/ml in a 1% w/w solution of propylene glycol in purified water) and perfluorobutane gas were homogenised in-line at 7800 rpm and ca. 40°C to yield a creamy-white microbubble
10 dispersion. The dispersion was fractionated to substantially remove undersized microbubbles ($<2\ \mu\text{m}$) and the volume of the dispersion was adjusted to the desired microbubble concentration by adding aqueous sucrose to
15 of the resulting dispersion were filled into 10 ml flat-bottomed vials specially designed for lyophilisation, and the contents were lyophilised to give a white porous cake. The lyophilisation chamber was then filled with
20 perfluorobutane and the vials were sealed. Prior to use, water was added to a vial and the contents were gently hand-shaken for several seconds to give a
perfluorobutane microbubble dispersion; the concentration of microbubbles in the dispersion was 1.1% v/v and the median microbubble size was $2.7\ \mu\text{m}$.

25

Preparation 2

Perfluorobutane gas dispersion with positively charged gas-stabilising material

30

1 ml of a dispersion of 1,2-distearoyl-3-trimethylammoniumpropane (1 mg/ml) and distearoylphosphatidylcholine (4 mg/ml) in a 2% w/v solution of propylene glycol in purified water was placed in a 2 ml vial. The
35 headspace was flushed with perfluorobutane gas and the vial was then closed and shaken for 45 seconds using an Espe CapMix® mixer for dental materials. The resulting

milky white microbubble dispersion was washed three times by centrifugation and removal of infranatant, whereafter an equal volume of purified water was added. The concentration of microbubbles in the resulting dispersion was 4.9% v/v and the median microbubble size was 3.2 μm .

Preparation 3

Perfluorobutane gas dispersion with biotinylated gas-stabilising material

Distearoylphosphatidylserine (4.5 mg) and biotin-dipalmitoylphosphatidylethanolamine (0.5 mg) were weighed into a clean vial and 1.0 ml of a solution of 1.4% propylene glycol/2.4% glycerol was added. Following heating to 78°C the mixture was cooled to room temperature and the head space was flushed with perfluorobutane gas. The vial was closed, shaken for 45 seconds using an Espe CapMix® mixer, and then placed on a roller table for 16 hours. The resulting microbubble dispersion was washed extensively with deionised water.

Preparation 4

25

Perfluorodimethylcyclobutane emulsion with positively charged emulsion-stabilising material

1 ml of a dispersion of didodecyldimethylammonium bromide (5 mg/ml in purified water) was placed in a 2 ml vial to which was added 100 μl of perfluorodimethylcyclobutane (b.p. 45°C). The vial was closed and then shaken for 75 seconds using an Espe CapMix® to yield an emulsion of diffusible component which was stored at 0°C when not in use. The emulsion was washed three times by centrifugation and removal of the infranatant followed by addition of an equivalent volume of purified water.

The concentration of droplets in the emulsion was 6.2% v/v and the median droplet size was 2.3 μm .

Preparation 5

5

Perfluorohexane emulsion with positively charged emulsion-stabilising material

10 1 ml of a dispersion of 1,2-distearoyl-3-trimethyl-
ammoniumpropane (1 mg/ml) and distearoylphosphatidyl-
choline (4 mg/ml) in purified water was placed in a 2 ml
vial to which was added 100 μl of perfluorohexane (b.p.
57°C). The vial was closed and then shaken for 75
15 seconds using an Espe CapMix® to yield an emulsion of
diffusible component which was stored at 0°C when not in
use. The emulsion was washed three times by
centrifugation and removal of the infranatant followed
by addition of an equivalent volume of purified water.
The concentration of droplets in the emulsion was 2.9%
20 v/v and the median droplet size was 2.9 μm .

Preparation 6

Perfluorodimethylcyclobutane emulsion with negatively 25 charged emulsion-stabilising material

1 ml of a dispersion of hydrogenated
phosphatidylserine (5 mg/ml in purified water) was
placed in a 2 ml vial to which was added 100 μl of
30 perfluorodimethylcyclobutane (b.p. 45°C). The vial was
closed and then shaken for 75 seconds using an Espe
CapMix® to yield an emulsion of diffusible component
which was stored at 0°C when not in use. The emulsion
was washed three times by centrifugation and removal of
35 the infranatant followed by addition of an equivalent
volume of purified water. The concentration of droplets
in the emulsion was 6.9% v/v and the median droplet size

was 2.7 μm .

Preparation 7

5 Perfluorodimethylcyclobutane emulsion with avidinylated emulsion-stabilising material

Distearoylphosphatidylserine (4.5 mg) and
10 biotin-dipalmitoylphosphatidylethanolamine (0.5 mg) were
weighed into a clean vial and 1.0 ml of a solution of 2%
propylene glycol was added. Following heating to 80°C
the mixture was cooled to room temperature. 100 μl of
perfluorodimethylcyclobutane were added and the vial was
closed and shaken for 75 seconds using an Espe CapMix®
15 to yield an emulsion of diffusible component. A diluted
sample of the emulsion (100 μl emulsion in 1 ml water)
was incubated with excess avidin and placed on a roller
table. The diluted emulsion was then washed extensively
with water and concentrated by centrifuging.

20

Example 1

In vivo imaging of dog heart

25 A 20 kg mongrel dog was anaesthetised, a mid-line
sternotomy was performed, and the anterior pericardium
was removed. Mid-line short-axis B-mode imaging of the
heart was performed through a low-attenuating 30 mm
silicone rubber spacer, using an ATL HDI-3000 scanner
30 equipped with a P3-2 transducer. The framerate was 40
Hz and the mechanical index was 1.1.

a) [Comparative] Imaging using negatively charged
perfluorobutane gas dispersion and negatively charged
35 perfluorodimethylcyclobutane emulsion

An amount of the perfluorobutane gas dispersion

from Preparation 1 corresponding to 0.2 μ l gas/kg body weight and an amount of the perfluorodimethylcyclobutane emulsion from Preparation 6 corresponding to 0.4 μ l perfluorodimethylcyclobutane/kg body weight were
5 simultaneously injected intravenously into the dog. A substantial rise in echo intensity from the myocardium was seen, starting 20 seconds after the injection and lasting for 10 minutes. Clearance of contrast agent effects from the blood pool occurred earlier than loss
10 of myocardial contrast effect.

b) Imaging using negatively charged perfluorobutane gas dispersion and positively charged perfluorodimethylcyclobutane emulsion (high dose)

15 An amount of the perfluorobutane gas dispersion from Preparation 1 corresponding to 0.2 μ l gas/kg body weight and an amount of the perfluorodimethylcyclobutane emulsion from Preparation 4 corresponding to 0.1 μ l
20 perfluorodimethylcyclobutane/kg body weight were simultaneously injected intravenously into the dog. The resulting myocardial contrast effect was far more intense than that observed in (a) above and lasted for
25 20 minutes.

c) Imaging using negatively charged perfluorobutane gas dispersion and positively charged perfluorodimethylcyclobutane emulsion (low dose)

30 The procedure described in Example 1(b) was repeated except that the dose of the perfluorodimethylcyclobutane emulsion was reduced to an amount corresponding to 0.02 μ l perfluorodimethylcyclobutane/kg body weight. The resulting myocardial contrast effect
35 was comparable to that observed in Example 1(a).

Example 2

In vivo imaging of dog heart (low contrast agent dose)

5

a) Imaging using negatively charged perfluorobutane gas dispersion and positively charged perfluorohexane emulsion

10

The procedure described in Example 1(b) was repeated except that the perfluoromethylcyclobutane emulsion was replaced by an amount of the perfluorohexane emulsion from Preparation 5 corresponding to 0.02 μ l perfluorohexane/kg body weight. The resulting myocardial contrast effect was comparable to that observed in Example 1(a).

15

20

b) [Comparative] Imaging using negatively charged perfluorobutane gas dispersion and negatively charged perfluorodimethylcyclobutane emulsion

25

The procedure described in Example 1(a) was repeated except that the dose of the perfluorodimethylcyclobutane emulsion was reduced to an amount corresponding to 0.02 μ l perfluorodimethylcyclobutane/kg body weight. Only faint myocardial contrast effects could be seen.

30

c) [Comparative] Imaging using positively charged perfluorobutane gas dispersion and positively charged perfluorohexane emulsion

35

An amount of the perfluorobutane gas dispersion from Preparation 2 corresponding to 0.2 μ l gas/kg body weight and an amount of the perfluorohexane emulsion from Preparation 5 corresponding to 0.02 μ l perfluorohexane/kg body weight were simultaneously

injected intravenously into the dog, imaging as in Example 1. Only faint myocardial contrast effects could be seen.

- 5 d) Imaging using positively charged perfluorobutane gas dispersion and negatively charged perfluorodimethylcyclobutane emulsion

10 An amount of the perfluorobutane gas dispersion from Preparation 2 corresponding to 0.2 μ l gas/kg body weight and an amount of the perfluorodimethylcyclobutane emulsion from Preparation 6 corresponding to 0.02 μ l perfluorohexane/kg body weight were simultaneously injected intravenously into the dog, imaging as in
15 Example 1. The resulting myocardial contrast effect was comparable to that observed in Example 1(a).

Example 3 [Comparative]

- 20 Imaging of dog heart using positively charged perfluorohexane emulsion only

 An amount of the perfluorohexane emulsion from Preparation 5 corresponding to 0.02 μ l perfluorohexane/kg body weight was injected intravenously into the dog,
25 imaging as in Example 1. No blood pool or myocardial contrast effects could be seen.

Example 4 [Comparative]

- 30 Imaging of dog heart without preliminary ultrasonication

 The procedure of Example 1(c) was repeated except that the ultrasound scanner was switched off for the
35 first 2 minutes following injection. The contrast effect in the myocardium after the scanner was switched on again was very brief, comparable to that seen at the

same time and with the same imaging modality following injection of perfluorobutane gas dispersion alone.

Example 5

5

Imaging of dog heart using biotinylated perfluorobutane gas dispersion and avidinylated perfluorodimethylcyclobutane emulsion

10 An amount of the perfluorobutane gas dispersion from Preparation 3 corresponding to 0.02 μ l gas/kg body weight and an amount of the perfluorodimethylcyclobutane emulsion from Preparation 7 corresponding to 0.02 μ l perfluorodimethylcyclobutane/kg body weight were
15 simultaneously injected intravenously into the dog.

 Imaging of the heart was performed with a Vingmed CFM-750 sanner, using a midline short axis projection. The scanner was adjusted to maximise ultrasound exposure to the imaged tissue region by using a combination of
20 continuous high frame rate imaging and the highest output power (7 on a scale ranging from 0 to 7). After the injection, initial contrast enhancement was seen in both ventricles of the heart. A steady rise in contrast enhancement was seen in all regions of the myocardium,
25 up to an enhancement intensity approaching the maximum white level on the screen. The duration of tissue contrast was approximately 30 minutes, whilst contrast effects in the blood-pool declined to near baseline within 5 minutes of the injection, leaving an image with
30 almost no blood-pool attenuation, and a complete and extremely bright circumferential contrast enhancement of the myocardium. The contrast effect in the myocardium close to the transducer did not seem to fade despite continuous high intensity ultrasound exposure.